(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 May 2003 (15.05.2003)

PCT

(10) International Publication Number
WO 03/039470 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/US02/35625

(22) International Filing Date: 7 November 2002 (07.11.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/331,036 7 November 2001 (07.11.2001) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/039470 A2

(54) Title: POLYVALENT IMMUNOGEN

(57) Abstract: The present invention relates, generally, to a polyvalent immunogen and, more particularly, to a method of inducing neutralizing antibodies against HIV and to a polyvalent immunogen suitable for use in such a method.

POLYVALENT IMMUNOGEN

This application claims priority from Provisional Application No. 60/331,036, filed November 7, 2001, the content of which is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates, generally, to a polyvalent immunogen and, more particularly, to a method of inducing neutralizing antibodies against HIV and to a polyvalent immunogen suitable for use in such a method.

BACKGROUND

Immunogenic peptides have been developed that elicit B and T cell responses to various strains of human immunodeficiency virus (HIV) (Palker et al, J. Immunol. 142:3612-3619 (1989), Haynes et al, Trans. Am. Assoc. Physician 106:31-41 (1993), Haynes et al, J. Immunol. 151:1646-1653 (1993), Haynes et al, AID Res. Human Retroviruses 11:211-221 (1995)) (see also WO 97/14436). These peptides consist of two components, each derived from noncontiguous regions of the HIV gp120 envelope protein. One envelope component consists of 16 amino acid residues from the fourth constant (C4) domain of HIV gp120, and includes a T-helper epitope (Cease et al, Proc. Natl. Acad. Sci. USA 84:4249-4253 (1987)). Linked to the carboxyl terminus of this gp120 C4 region peptide is a 23 amino acid segment from the third variable (V3) domain of gp120, that includes a B cell neutralizing antibody epitope for cell line-adapted HIV strains (Palker et al, J. Immunol. 142:3612-3619 (1989), (Palker et al, Proc. Natl. Acad. Sci. USA 85:1932-1936 (1988), Rusche et al, Proc. Natl. Acad. Sci. USA 85:3198-3202)), a T-helper epitope (Palker et al, J. Immunol. 142:3612-3619 (1989)), and two cytotoxic T lymphopoietic (CTL) epitopes (Clerici et al, J. Immunol. 146:2214-2219 (1991), Safrit et al, 6th NCVDG Meeting, Oct. 30 to Nov. 4, 1993)). In mice

and rhesus monkeys, these C4-V3 hybrid peptides have induced antibodies that bind to native gpl20 and neutralize the particular cell line-adapted strain of HIV from which the V3 segment was derived, as well as induce T helper cell proliferative responses and MHC Class I-restricted CTL responses that kill HIV or HIV protein expressing target cells (Palker et al, J. Immunol. 142:3612-3619 (1989), Haynes et al, AID Res. Human Retroviruses 11:211-221 (1995)). Recently, it was shown that this gpl20 peptide design can induce antibodies that neutralize primary HIV isolates and simian-human immunodeficiency viruses (SHIV) expressing primary HIV isolate envelopes (Liao et al, J. Virol. 74:254-263 (2000)). Moreover, in a challenge trial of this immunogen in rhesus monkeys, it was shown that C4-V3 peptides from the gpl20 of the pathogenic SHIV 89.6P, induced neutralizing antibodies that prevented the fall in CD4 counts after challenge with SHIV 89.6P (Letvin et al, J. Virol. 75:4165-4175 (2001)). Therefore, anti-V3 antibodies can protect primates against primary isolate SHIV-induced disease.

A prototype polyvalent HIV experimental immunogen comprised of the conserved C4 region of gpl20 and the V3 regions of HIV isolates MN, CANO(A), EV91 and RF has been constructed and has been found to be highly immunogenic in human clinical trials (Bartlett et al, AIDS 12:1291-1300 (1998), Graham et al, Abstract, AIDS Vaccine (2001)). Thus, understanding secondary and higher order structures of the components of this polyvalent immunogen, as well as defining strategies to optimize gpl20 immunogen antigenicity, is important to HIV vaccine design efforts. In addition, recent data suggest that the HIV V3 region may be involved in regulating gpl20 interactions with HIV co-receptors, CXC chemokine receptor 4 (CXCR4) and 13 chemokine receptor type 5 (CCR5) (Berger, AIDS Suppl. A:53-56 (1997)).

In previous studies, nuclear magnetic resonance (NMR) has been used to characterize conformations of the multivalent immunogen C4-V3 peptides in solution (de Lorimier et al, Biochemistry 33:2055-2062 (1994), Vu et al,

Biochemistry 35:5158-5165 (1996), Vu et al, J. Virol. 73:746-750 (1999)). It as been found that the V3 segments of each of the four C4-V3 peptides displayed evidence of preferred solution conformations, with some features shared, and other features differing among the four peptides. The C4 segment, which is of identical sequence in all the peptides, showed in each case a tendency to adopt nascent helical conformations (de Lorimier et al, Biochemistry 33:2055-2062 (1994), Vu et al, Biochemistry 35:5158-5165 (1996), Vu et al, J. Virol. 73:746-750 (1999)).

The C4 domain of gpl20 is part of the CD4 binding site, and antibodies recognizing the C4 sequence, block the binding of CD4 of gpl20. However the C4 sequence as a peptide does not elicit antibodies that bind native gpl20 (Palker et al, J. Immunol. 142:3612-3619 (1989), Haynes et al, J. Immunol. 151:1646-1653 (1993), Ho et al, J. Virol. 61:2024-2028 (1987), Robey et al, J. Biol. Chem. 270:23918-23921 (1995)). This led to the speculation that the nascent helical conformations exhibited by the C4 segment might reflect a conformation not native to HIV gpl20. Amino-acid sequence homology between the gp120 C4 region and a human IgA CH1 domain has been noted (Maddon et al, Cell 47:333-348 (1986)). By comparison to the structure of mouse IgA (Segal et al, Proc. Natl. Acad. Sci. USA 71:4298-4302 (1974)), the C4-homologous region of IgA has a β strand secondary structure (de Lorimier et al, Biochemistry 33:2055-2062 (1994)). Therefore, while the C4 gpl20 peptide in solution adopts nascent helical conformations, the native structure of this gpl20 C4 region may be quite different (ie, in the context of gp 120 have a β strand secondary structure).

The present invention results, at least in part, from the results of a study with a three-fold purpose. First, C4-V3HIVRF peptides with amino acid substitutions designed to minimize C4 α -helical peptide conformation and promote β strand C4 secondary structures were constructed in order to induce anti-native gpl20 antibodies with the modified C4 peptide. Second, tests were made to determine if any of these mutated C4-V3RF peptides would enhance

gpl20 V3 region peptide immunogenicity, and therefore augment anti-HI VRF gpl20 V3 loop antibody responses. Finally, the solution conformers of each peptide studied immunologically were also solved using NMR to correlate peptide conformers with peptide immunogenicity.

SUMMARY OF THE INVENTION

The present invention relates to a method of inducing neutralizing antibodies against HIV and to peptides, and DNA sequences encoding same, that are suitable for use in such a method.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Summary of antibody binding titers to immunizing peptide after 2 or 3 boosts of 3 mice in each group with immunizing peptide. There was a slight enhancement of levels of antibody induced by the E9G variant after 2 but not 3 boosts, while the E9V variant significantly boosted antibody levels compared to the C4-V3RF(A) peptide after 2 and 3 boosts. Antibody to the K12E variant induced by the K12E peptide was significantly lower than C4-V3RF(A) induced antibody levels after both 2 and 3 boosts.

Figure 2: NMR spectra of the four C4-V3RF variant peptides.

Figure 3: $C4_{E9V}$-V389.6 peptides bound better to human PB lymphocytes and monocytes than did the C4-V3 89.6 peptides. Similar data were obtained with the C4-V3 89.6P and C4-E9V-89.6P peptides. Sequence of the C4-V389.6 peptide form HIV89.6 isolate was: KQIINMWQEVGKAMYA-TRPNNNTRRRLSIGPGRAFYARR; the sequence of the $C4_{E9V}$-V389.6 peptide was: KQIINMWQVVGKAMYA-

TRPNNNTRRRLSIGPGRAFYARR; the sequence of the C4-V389.6P peptide was: KQIINMWQEVGKAMYA-TRPNNNTRERLSIGPGRAFYARR; the sequence of the C4E9V-V389.6P peptide was: KQIINMWQVVGKAMYA-TRPNNNTRERLSIGPGRAFYARR.

Figure 4: Neutralization of BAL in PBMC.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a composition comprising a multiplicity of immunogenic hybrid peptides, each comprising two components. One component includes a T-helper epitope and can comprise residues from the C4 domain of HIV gp120. The second component comprises residues from the V3 domain of gp120 and includes a B cell neutralizing antibody epitope.

Advantageously, the first component comprises about 16 contiguous residues from the C4 domain (about residues 421 to 436) and the second component comprises about 23-25 contiguous residues from the V3 domain (about residues 297 to 322). The components can, however, be longer or shorter. Preferably, the V3 component is linked C terminal to the C4 component peptide. The hybrid peptides can include additional sequences (e.g., linkers (e.g., cysteine, serine or lysine linkers) between the C4 and V3 components). The composition can, for example, comprise 5 to 10 hybrid peptides, 10 to 15 hybrid peptides or 25 to 30 hybrid peptides. The number of hybrid peptides used will depend, at least in part, on the target population.

Preferred first components comprising residues from the C4 domain are shown in the Tables that follow (see particularly Tables 6 and 7). Other T helper determinants from HIV or from non-HIV proteins can also be used. For example, a further T helper epitope suitable for use in the invention is from HIV gag (e.g., residues 262-278). One such sequence, designated

GTH1, is YKRWIILGLNKIVRMYS (from HIV p24 gag). Variants of this sequence can also be used. Alternatively, a carbohydrate such as the outer membrane protein of pneumococcus, or another carbohydrate or protein with immunogenic, T helper activity can be used.

The V3 components of the hybrid peptides present in the instant composition are selected so as to be representative of higher order structural motifs present in a population, which motifs mediate V3 functions in the course of envelope mediated HIV interaction with host cells. The Los Alamos National Laboratories Human Retroviruses and AIDS Database (Human Retroviruses and AIDS, 2000, Published by the Theoretical Biology and Biophysics G T-10, Mail Stop K710, LANL, Los Alamos, NM) presently contains over 14,000 HIV V3 envelope sequences, showing the extraordinary diversity the virus has obtained since originating in man in Africa approximately 50 years ago. For example, among 432 HIV-1 V3 sequences derived from individuals infected with subtype C (designated "Clade C") in Africa currently available in the HIV database, 176 distinct variants of a 23 amino acid stretch at the tip of the V3 loop have been found. Similarly, among 6870 B subtype (designated "Clade B") V3 sequences from the US, 1514 unique forms have been found.

A method has been developed to organize short antigenic domains by protein similarity scores using maximum-linkage clustering. This method enables the visualization of the clustering patterns as a dendrogram, and the splitting patterns in the dendrogram can be used to define clusters of related sequences (Korber et al, J. Virol. 68:6730-6744 (1994)). The method allows the use of several different amino acid similarity scoring schemes available in the literature, preferred is the amino acid substitution matrix developed by Henikoff and Henikoff (see Advances in Protein Chemistry 54:73-97 (2000) and Proteins: Structure, Function and Genetics 17:49-61 (1993)), designed to give substitutions that are well tolerated in conserved protein structural elements a high score, and a low score to those that are not. Typically

excluded from consideration very rare, highly divergent peptides, and favored are peptides found in many individuals within the population. In a selected set of sequences , most of the unique forms are within one or two amino acids from a least one other of the peptides chosen. This method has been applied to clustering the large number of variants of the antigenic tip of the V3 domain within Clade B and Clade C into groups (about 25) that are likely to be cross-reactive within the group. Based on these clustering patterns, variants (e.g., about 25-30) are selected that are representative or "central" to each group, for testing for antigenicity. The HIV Clade B and Clade C gp120 envelope V3 sequences have been analyzed, as described above, for groups of V3 sequences predicted to have structural similarities. Twenty five Clade C and 30 Clade B groups have been defined, and chosen out of each group is a common, or the most common, sequence as a representative of that group. The selected V3 sequences have been included in a C4-V3 design thereby providing a 25 peptide Clade C immunogen, and a 30 peptide Clade B immunogen (see Tables 6 and 7).

Table 6

C4-V3 design of Clade C V3 sequences

C4-V3-C1 **KQIINMWQVVGKAMYA**-trpnnntrksirigpGqtfyatg
C4-V3-C2 **KQIINMWQVVGKAMYA**-trpnnntrksirigpGqtfyaRg
C4-V3-C3 **KQIINMWQVVGKAMYA**-trpnnntrksirigpGqtfyaAg
C4-V3-C4 **KQIINMWQVVGKAMYA**-IrpnnntrksVrigpGqtfyatg
C4-V3-C5 **KQIINMWQVVGKAMYA**-trpnnntrksirigpGqtfFatg
C4-V3-C6 **KQIINMWQVVGKAMYA**-trpnnntrksirigpGqtfyatN
C4-V3-C7 **KQIINMWQVVGKAMYA**-trpnnntrEsirigpGqtfyatg
C4-V3-C8 **KQIINMWQVVGKAMYA**-trpnnntrRsirigpGqAfyatg
C4-V3-C9 **KQIINMWQVVGKAMYA**-trpnnntrkGirigpGqtfyatg
C4-V3-C10 **KQIINMWQVVGKAMYA**-trpSnntrksirigpGqAfyatg
C4-V3-C11 **KQIINMWQVVGKAMYA**-trpSnntrksirigpGqtfyatN

C4-V3-C12 **KQIINMWQVVGKAMYA**-trpSnntrEsirigpGqtfyatg
C4-V3-C13 **KQIINMWQVVGKAMYA**-trpnnntrksMrigpGqtfyatg
C4-V3-C14 **KQIINMWQVVGKAMYA**-trpGnntrksMrigpGqtfyatg
C4-V3-C15 **KQIINMWQVVGKAMYA**-trpGnntrksirigpGqtLyatg
C4-V3-C16 **KQIINMWQVVGKAMYA**-VrpnnntrksVrigpGqtSyatg
C4-V3-C17 **KQIINMWQVVGKAMYA**-trpGnntrRsirigpGqtfyatg
C4-V3-C18 **KQIINMWQVVGKAMYA**-IrpGnntrksVrigpGqtfyatg
C4-V3-C19 **KQIINMWQVVGKAMYA**-trpnnntrksirigpGqAfyatN
C4-V3-C20 **KQIINMWQVVGKAMYA**-trpnnntrQsirigpGqAfyatK
C4-V3-C21 **KQIINMWQVVGKAMYA**-trpGnntrksirigpGqAfFatg
C4-V3-C22 **KQIINMWQVVGKAMYA**-trpGnntrksVrigpGqAfyatN
C4-V3-C23 **KQIINMWQVVGKAMYA**-trpnnntrkGiHigpGqAfyaAg
C4-V3-C24 **KQIINMWQVVGKAMYA**-trpnnntrkGiGigpGqtfFatE
C4-V3-C25 **KQIINMWQVVGKAMYA**-trpGnntrEsiGigpGqAfyatg

Table 7

C4-V3 peptides Clade B

C4-V3-396.2 **KQIINMWQVVGKAMYA**-RPNNNTRRNIHIGLGRRFYAT-*
C4-V3-170.6 **KQIINMWQVVGKAMYA**-RPNNNTRRSVRIGPGGAMFRTG*
C4-V3-82.15 **KQIINMWQVVGKAMYA**-RPNNNTRRSIPIGPGRAFYTTG*
C4-V3-144.8 **KQIINMWQVVGKAMYA**-RPDNNTVRKIPIGPGSSFYTT-*
C4-V3-23.38 **KQIINMWQVVGKAMYA**-RPIKIERKRIPLGLGKAFYTTK*
C4-V3-365.2 **KQIINMWQVVGKAMYA**-RPSNNTRKGIHLGPGRAIYATE*
C4-V3-513.2 **KQIINMWQVVGKAMYA**-RPSNNTRKGIHMGPGKAIYTTD*
C4-V3-1448.1 **KQIINMWQVVGKAMYA**-RPGNTTRRGIPIGPGRAFFTTG*
C4-V3-69.18 **KQIINMWQVVGKAMYA**-RPNNNTRKSIRIGPGRAVYATD*
C4-V3-146.8 **KQIINMWQVVGKAMYA**-RPGNNTRRRISIGPGRAFVATK*
C4-V3-113.1 **KQIINMWQVVGKAMYA**-RPNNNTRRSIHLGMGRALYATG-*
C4-V3-51.23 **KQIINMWQVVGKAMYA**-RPSNNTRRSIHMGLGRAFYTTG-*
C4-V3-72.18 **KQIINMWQVVGKAMYA**-RPNNNTRKGINIGPGRAFYATG-*
C4-V3-36.29 **KQIINMWQVVGKAMYA**-RPNNNTRKGIHIGPGRTFFATG-*
C4-V3-70.18 **KQIINMWQVVGKAMYA**-RPNNNTRKRIRIGHIGPGRAFYATG*
C4-V3-89.14 **KQIINMWQVVGKAMYA**-RPSINKRRHIHIGPGRAFYAT-*
C4-V3-163.7 **KQIINMWQVVGKAMYA**-RLYNYRRKGIHIGPGRAIYATG*
C4-V3-57.20 **KQIINMWQVVGKAMYA**-RPNRHTGKSIRMGLGRAWHTTR*
C4-V3-11.85 **KQIINMWQVVGKAMYA**-RPNNNTRKSINIGPGRAFYTTG---*
C4-V3-34.29 **KQIINMWQVVGKAMYA**-RPNNNTRKSIQIGPGRAFYTTG---*
C4-V3-1.481 **KQIINMWQVVGKAMYA**-RPNNNTRKSIHIGPGRAFYTTG---*
C4-V3-85.15 **KQIINMWQVVGKAMYA**-RPNNNTRKSIHIAPGRAFYTTG---*
C4-V3-62.19 **KQIINMWQVVGKAMYA**-RPNNNTRKSIHIGPGRAFYATE------*
C4-V3-125.9 **KQIINMWQVVGKAMYA**-RPNNNTRRRISMGPGRVLYTTG*
C4-V3-35.29 **KQIINMWQVVGKAMYA**-RPNNNTRKRISLGPGRVYYTTG*
C4-V3-74.17 **KQIINMWQVVGKAMYA**-RPNNNTRKRMTLGPGKVFYTTG*
C4-V3-46.26 **KQIINMWQVVGKAMYA**-RPDNTIKQRIIHIGPGRPFYTT-*
C4-V3-122.9 **KQIINMWQVVGKAMYA**-RPNYNETKRIRIHRGYGRSFVTVR*
C4-V3-162.7 **KQIINMWQVVGKAMYA**-RPGNNTRGSIHLHPGRKFYYSR*
C4-V3-3.323 **KQIINMWQVVGKAMYA**-RPNNNTRKSINMGPGRAFYTTG

While the above is offered by way of example, it will be appreciated that the same analyses can by performed for HIV Clades A, D, E, F, G, H, M, N, O, etc, to design V3 immunogens that react with HIV primary isolates from these Clades.

In addition to the sequences described in Tables 6 and 7, a substitution has been made in the C4 sequence at position 9 from E to V to enhance the binding of the C4 region to human immune cell membranes, and to increase immunogenicity (see Example that follows). Substituting V for E at position 9 of C4 results in the C4-E9V-V3RF(A) peptide inducing 2-3 logs higher anti-gp 120 V3 region antibody levels compared with the original C4-V3RFA(A) peptide. The effect of the E9V substitution is not species specific. While not wishing to be bound by theory, the data may indicate that the ability of the E9V variant peptide to enhance B cell antibody production is not MHC specific but rather it relates in some manner to non-MHC specific factors, such as the ability of the peptides to bind to the lipid bilayer of immune cells. The data presented in Figure 3 demonstrate the ability of $C4_{E9V}$-V389.6 peptides to bind to human PB lymhocytes and monocytes. The ability of the C4 and C4E9V "T helper" determinants to facilitate immunogenicity of the V3 region may be due to the ability of helical amphipathic structures to interact with lipid bilayers in a non-MHC related manner and promote peptide internalization. The invention encompasses the use of C4 sequences in addition to those described above.

The peptide immunogens of the invention can be chemically synthesized and purified using methods which are well known to the ordinarily skilled artisan. (See, for example, the Example that follows.) The composition can comprise the peptides linked end to end or can comprise a mixture of individual peptides. The peptide immunogens can also be synthesized by well-known recombinant DNA techniques. Recombinant synthesis may be preferred when the peptides are covalently linked.

Nucleic acids encoding the peptides of the invention can be used as components of a DNA vaccine wherein the peptide encoding sequence(s) is/are administered as naked DNA or, for example, a minigene encoding the peptides can be present in a viral vector, such as an adenoviral vector, a modified vaccinia ankara vector, a vaccinia vector or an attenuated TB vector. Expression of the immunogenic peptides of the invention can be induced in a patient's own cells, by introduction into those cells of nucleic acids that encode the peptides, preferably using codons and promoters that optimize expression in human cells. Examples of methods of making and using DNA vaccines are disclosed in U.S. Pat. Nos. 5,580,859, 5,589,466, and 5,703,055.

The composition of the invention comprises an immunologically effective amount of the peptide immunogens of this invention, or DNA sequence(s) encoding same, in a pharmaceutically acceptable delivery system. The compositions can be used for prevention and/or treatment of immunodeficiency virus infection. The compositions of the invention can be formulated using adjuvants, emulsifiers, pharmaceutically-acceptable carriers or other ingredients routinely provided in vaccine compositions. Optimum formulations can be readily designed by one of ordinary skill in the art and can include formulations for immediate release and/or for sustained release, and for induction of systemic immunity and/or induction of localized mucosal immunity (e.g, the formulation can be designed for intranasal administration). The present compositions can be administered by any convenient route including subcutaneous, intranasal, oral, intramuscular, or other parenteral or enteral route. The immunogens can be administered as a single dose or multiple doses. Optimum immunization schedules can be readily determined by the ordinarily skilled artisan and can vary with the patient, the composition and the effect sought. By way of example, it is noted that approximately $50\mu g$-$100\mu g$ of each hybrid peptide can be administered, for example, intramuscularly (e.g. 3x).

The invention contemplates the direct use of both the peptides of the invention and nucleic acids encoding same. For example, a minigene encoding the peptides can be used as a prime and/or boost.

In addition to the composition described above, the invention encompasses each of the hybrid peptides disclosed as well as each of the components (C4 and V3), alone or in covalent or non-covalent association with other sequences. The invention further encompasses nucleic acid sequences encoding any and all such peptides.

Certain aspects of the invention can be described in greater detail in the non-limiting Example that follows.

EXAMPLE 1

Experimental Details

Peptide design, synthesis and purification.

Peptides were designed, as shown in Table 1. It was hypothesized that alteration of the C4 sequence to reduce its helical conformational tendency in peptides might cause enrichment of solution conformers resembling a β strand conformation. This in turn might cause C4 to be immunogenic for antibodies recognizing the native conformation of the C4 (part of the CD4 binding site) region of gpl20. The present work describes tests of this hypothesis in chimeric peptide C4-V3 RF, which has a V3 segment from gpl20 of HIV strain RF, and three sequence variants wherein single amino-acid replacements have been introduced at position 9 in the C4 segment, Glu (E) to Gly (G), Glu (E) to Val (V), and at position 12, Lys (K) to Glu (E) (Table 1). These replacements were made in part to disrupt possible stabilization of helical conformations due to side-chain *(i, i+3)* charge interaction between E9 and K12 (Scholtz et al, Biochemistry 32:9668-9676 (1993)). In addition, the substitution in $C4_{E9G}$-V3RF(A) was expected to disfavor helix formation by introducing greater main-chain flexibility (Chakrabartty et al, Adv. Protein Chem. 46:141-176 (1995)). Furthermore the substitution in $C4_{E9G}$-V3RF(A)

introduced two adjacent valine residues which has been hypothesized to favor extended conformations. Thus, the parent peptide, C4-V3RF(A) (Haynes et al, AID Res. Human Retroviruses 11:211-221 (1995)) contained 16 N-terminal residues from the C4 domain of $gp120_{IIIB}$ and 23 C-terminal residues from the V3 domain of gpl20 of HIVRF.

TABLE 1

Peptides Used in This Study

Peptide	Sequence	
	C4	V3
	1 16	17 39
C4-V3RF(A)	KQIINMWQEVGKAMYA	TRPNNNTRKSITKGPGRVIYATG
$C4_{E9G}$-V3RF(A)	KQIINMWQGVGKAMYA	TRPNNNTRKSITKGPGRVIYATG
$C4_{E9V}$-V3RF(A)	KQJINMWQVVGKAMYA	TRPNNNTRKSITKGPGRVIYATG
$C4_{K12E}$-V3RF(A)	KQIIINMWQEVGEAMYA	TRPNNNTRKSITKGPGRVIYATG

All sequences from Los Alamos National Laboratory AIDS Sequence Database.

Peptides were synthesized by fluorenylmethoxycarbonyl chemistry on an ABI 43 lA peptide synthesizer (Applied Biosystems, Inc., Foster City, CA), then purified by reverse-phase high performance liquid chromatography. The purity and identity of the product were confirmed by determining molecular mass by electrospray mass spectrometry.

Immunization methods.

Mice were immunized with 50μg of the indicated peptide in incomplete Freund's adjuvant (1SA51, Seppic Inc., Paris France) at weeks 0, 3, and 7 and bled at weeks 2, (bleed 1 after boost 1), week 5 (bleed 2 after boost 2) and week 8 (bleed 3 after boost 3). Immune responses were seen after bleed 2 in most animals and data are reported from bleeds 2 and 3.

Guinea pigs were immunized intranasally with 200μg of C4-V3 peptide in saline with 1μg of cholera toxin as adjuvant as described. Guinea pigs were immunized on day 0, day 14 and day 21 and serum samples before and 1 week following each immunization obtained by cardiac puncture.

ELISA Assay.

Anti-HIV env peptide ELISA assays were performed as previously described (Haynes et al, J. Immunol. 151:1646-1653 (1993), Haynes et al, AID Res. Human Retroviruses 11:211-221 (1995)).

Splenocyte Proliferation Assay.

Mouse splenocyte proliferation assay using ^{3}H-thymidine incorporation was performed as previously described (Haynes et al, AID Res. Human Retroviruses 11:211-221 (1995)).

Neutralizing Antibody Assays.

Assays for ability of anti-HIV antisera to neutralize HIV were performed as described (Palker et al, J. Immunol. 142:3612-3619 (1989), Haynes et al, Trans. Am. Assoc. Physician 106:31-41 (1993), Haynes et al, J.

Immunol. 151:1646-1653 (1993), Haynes et al, AID Res. Human Retroviruses 11:211-221 (1995)).

NMR spectroscopy.

Peptides were dissolved to 4 mM in a solution of 90% $^{1}H_{2}0$, 10% $^{2}H_{2}0$, 20 mM NaCl, 5 mM KH_2PO_4, 1 mM sodium azide, 0.5 mM sodium 3-(trimethylsilyl) propionate, at a pH of 4.2. The methyl resonance of the latter component served as a chemical shift reference.

Spectra of samples prepared in this way were acquired with a Varian Unity 500 MHz spectrometer at a temperature of 278 K. The lock signal was from deuterium in the sample. The following two-dimensional spectra were obtained: (a) double-quantum-filtered correlation spectroscopy (DQF-COSY) (Piantini et al, J. Am. Chem. Soc. 104:6800-6801 (1982), Rance et al, Biochem. Biopjys. Res. Commun. 117:479-485 (1983)); (b) total correlation spectroscopy (TOCSY) (Bax et al, J. Magn. Reson. 65:355-360 (1985), Levitt et al, J. Magn. Reson. 47:328-330 (1982)) with a mixing time of 150 ins; and (c) nuclear Overhauser exchange spectroscopy (NOESY) (Jeener et al, J. Phys. Chem. 71:4546-4553 (1979)) with a mixing time of 300 ins. Water resonance was suppressed by selective saturation during the relaxation delay, and, for NOESY, during the mixing period. The spectral width was 6700 Hz, with the indirectly acquired dimension collected as 750 (COSY), 512 (TOCSY), or 350 (NOESY) complex increments; and the directly acquired dimension containing 1024 complex points. Data were processed with FELIX 2.3 software (Biosym, San Diego, CA). Directly acquired free-induction decays were corrected for base-line offset. Decays in both dimensions were multiplied by a sinebell-squared function (phase shifted by 75°) and zero-filled to 2048 points before Fourier-transformation.

Peptide Membrane Binding Assay.

Peptides at 100ng/ml were incubated with 106 peripheral blood mononuclear cells for 1 hour at 4^{0}C, washed x3 with phosphate buffered saline

PHz 7.0, contained 0.1% sodium azide, then incubated guinea pig anti-HIV 89.6 V3 antisera (xlhr) (Liao et al, J. Virol. 74:254-263 (2000)), wash as above and then incubated with FITC-conjugated goat anti-guinea pig IgG. After a final wash as alone, the cells were analyzed for the relative amount of peptide bound to either PB lymphocytes or PB monocytes as reflected in the mean fluorescent channel (MFC) of reactivity of the anti-HIV 89.6 V3 antisera.

Results

Anti-gpl20 V3 Antibody Responses Following Immunization of Mice With C4-V3RF, $C4_{E9V}$-V3RF(A), $C4_{E9G}$- V3RF(A) and $C4_{K12E}$- V3RF(A) Peptides.

First, the ability of C4-V3HIVRF variants to modulate the immunogenicity of the peptide with regard to antibodies to the V3 portion of the C4-V3 immunogen were assayed. The results (Figure 1, Table 2) show differences among the four peptides in their ability to induce anti-HIVRF V3 antibody responses. Sera from $C4_{E9V}$-V3RF(A)-immunized mice had a log higher anti-V3 antibody titer than either mice immunized with the native C4-V3RF(A) peptide or the C4E9V-V3RF(A) peptide variant. After one immunization, no anti-V3RF antibody response was seen in mice immunized with either C4-V3RF(A), $C4_{E9G}$-V3RF(A), or $C4_{K12E}$-V3RF(A) peptides. However, after only one immunization with 50μg of the $C4_{E9V}$-V3 peptide, the geometric mean titer to V3RF(A) peptide was 1:5012 (n =3 mice), with titers of 1:3200, 1:3200 and 1:12,800 in each of the three mice tested, respectively. Thus, the E9V C4-V3RF(A) variant induced a higher titer and earlier anti-gp 120 V3 antibody responses than the other C4-V3RF(A) peptides tested. After 2 boosts, $C4_{E9V}$-V3RF(A)-immunized mice had 2 logs higher anti-V3 antibody responses than did C4-V3RF(A) immunized mice (Figure 1, Table 2).

TABLE 2

Comparison of the Ability of C4-V3 Peptides To Induce HIV gp120 Anti-C4 and Anti-V3 Antibodies in Balb/c Mice

Peptide Immunogen	Number of Animals	Peptide on Plate in ELISA For Anti-Peptide Antibody Assay					
		C4	V3RF(A)	C4-V3RF(A)	C4E9G-V3RF(A)	C4E9V-V3RF(A)	C412EV3RF(A)
	Geometric Mean Titer						
C4-V3RF(A)	6	2	1,584	2,239	1,195	1,584	1,412
$C4_{E9G}$-V3RF(A)	6	2	6,310	7,079	5,623	3,162	3,548
$C4_{E9V}$-V3RF(A)	5	14	151,356	131,825	87,096	87,096	114,815
$C4_{K12E}$-V3RF(A)	6	1	8	8	1	3	3

Data represent the reciprocal of endpoint dilutions at which the E/C was $\geq$ 3.0 in anti-peptide ELISA after two immunizations.

The $C4_{K12E}$-V3RF(A) peptide variant induced anti-V3 antibody responses 3 logs lower than the C4-V3RF(A) peptide after 2 immunizations (Figure 1, Table 2). Thus, single amino-acid replacements in the C4 T helper region had extraordinary effects on immunogenicity of the HIVRF gpl20 V3 domain.

Comparison of the Ability of C4-V3RF(A) Peptides to Induce Anti-HIVgpl20 Peptide 3H-Thymidine Incorporation in Splenocytes From Naive and Peptide-Immunized Mice.

Next, C4-V3 peptides were tested for their ability to stimulate proliferation of splenocytes from peptide-immunized mice. Balb/c mice were sacrificed after the third peptide immunization and their splenocytes assayed for the ability to proliferate to PHA and to each peptide type (Table 3). It was found that C4-V3RF(A), $C4_{E9V}$-V3RF(A), and $C4_{K12E}$-V3RF(A) peptides all induced *in vitro* proliferative responses to the immunizing peptides, whereas the $C4_{E9G}$-V3RF(A) variant peptide did not induce proliferative responses in E9G-primed mice significantly over responses of naive mice (Table 3). Regarding the ability of the E9V peptide variant to induce earlier and greater anti-V3 antibody responses compared to the other peptides tested, the $C4_{E9V}$-V3RF(A) peptide-primed splenocytes for proliferation to the immunizing peptide only minimally better than did each of the other three peptides (Table 3). Thus, altered induction of T helper cell proliferative responses did not explain the differences in peptide immunogenicity.

TABLE 3

Comparison of the Ability of C4-V3 Peptides To Induce Anti-HIV gp120 Peptide ^{3}H-Thymidine Incorporation in Splenocytes from Naïve and Immunized Mice

Peptide Immunogen	Peptide Used As Stimulator in ^{3}H-Thymidine Incorporation Assay						
	N	C4	V3RF(A)	C4-V3RF(A)	$C4_{E9G}$-V3RF(A)	$C4_{E9V}$-V3RF(A)	$C4_{K12E}$-V3RF(A)
	Mean ± SEM Δ CPM per 10^6 Splenocytes in Culture						
None (Naïve Balb/c)	6	613 ± 322	408 ± 140	149 ± 84	114 ± 85	74 ± 47	187 ± 165
C4-V3RF(A)	6	2,289 ± 1,332	955± 353	8,390 ± 1,424 a	8,067 ± 1,728	6,242 ± 1,787	6,198 ± 1,343
$C4_{E9G}$-V3RF(A)	6	408 ± 95	708 ± 325	2,103 ± 1,170	3,559 ± 2,310 b	988 ± 340	1,101 ± 399
$C4_{E9V}$-V3RF(A)	5	84 ± 52	1,463 ± 473	933 ± 4,528	11,743 ± 3,830	24,824 ± 5,581 c	10,269 ± 3,592 [illegible]
$C4_{K12E}$-V3RF(A)	6	3,430 ± 2,796	4,417 ± 2,217	8,670 ± 3,865	13,237 ± 8,563	7,513 ± 2,951	12,644 ± 4,138 [illegible]

Data represent peak 3H-thymidine responses at 7 days.

Δ CPM = CPM experimental – experimental – experimental control.
[a]$p < .001$ vs naïve mice; p = NS vs C4-V3RF(A) or C4K12E-V3RF(A) stimulated C4K12E-V3RF(A) immunized splenocytes.
[b]p = NS vs naïve mice.
[c]$p < .001$ vs naïve mice.
[d]$p < .02$ vs naïve mice.

The lower antibody titer induced by the $C4_{K12E}$-V3 peptide against V3RF(A) was not an artifact attributable to lack of ability of the V3 peptide not binding to the ELISA plate, as sera from $C4_{E9V}$-V3RF(A)-induced antisera had high reactivity to the V3RF(A) peptide on the ELISA plate. Similarly, the $C4_{K12E}$-V3RF(A) peptide could bind anti-V3RF antibody, as multiple antisera raised against C4-V3 peptides bound the $C4_{K12E}$-V3 variant (Table 2).

Antibody levels to the C4 region were also tested. The C4 region induced only a minimal antibody response compared to the V3 region, with all the C4-V3 peptides tested (Table 2).

Anti-gp 120 V3 Antibody Responses Following Immunization of Guinea Pigs.

Next, 2 guinea pigs were immunized each with 200μg of C4-V3RF(A), $C4_{E9G}$-V3 RF(A), $C4_{E9V}$-V3 RF(A) or $C4_{K12E}$-V3 RF(A) peptide intranasally with 1μg cholera toxin adjuvant in saline. Intranasal immunization of peptides with cholera toxin has been previously shown to result in CTL and titers of anti-peptide antibody similar in levels to titers induced by initial antigens administered subcutaneously or intramuscularly in oil in water adjuvants such as complete and incomplete Freund's adjuvant. In addition, it was desirable to determine the ability of C4-V3 peptides in an aqueous solution (such as in saline for intranasal immunization) to induce anti-HIV antibody responses in order to correlate reactivity of antibodies generated against peptide in an aqueous adjuvant with peptide conformers solved in an aqueous solution. Finally, there was interest in determining if the amino acid substitutions in the C4 region conferred on the C4-V3 peptides the same pattern of immunogenicity as seen in oil in water adjuvant in mice.

It was found that after 2 immunizations the C4-V3 RF(A) peptide induced a mean anti-HIV peptide antibody titer of 3981, peptide induced titers of 1 log (GMT = 31,623) higher. As in mice, substituting the Glu (E) for Lys (K) at position 12 in the C4 peptide abrogated peptide immunogenicity in guinea pigs (GMT = 16) (Table 4).

TABLE 4

Titers of C4-V3 HIV Envelope Antibodies Induced by C4-V3RF(A) Peptides in Guinea Pigs

Immunizing Peptide	Titer Against Immunizing Peptide*
C4-V3RF(A)	3,981
C4-$_{E9G}$-V3RF(A)	2,818
C4-$_{E9V}$-V3RF(A)	31,623
C4-$_{K12E}$-V3RF(A)	16

*Data represent the mean titers from 2 animals after 2-3 immunizations intranasally with 400ug of the indicated peptide formulated in saline with cholera toxin as an adjuvant.

Ability of Antibodies Against C4- V3 Peptides to Induce Neutralizing Antibodies.

In order to induce high levels of neutralizing antibodies with C4-V3 peptides, usually *5* immunizations are given (Palker et al, J. Immunol. 142:3612-3619 (1989), Haynes et al, J. Immunol. 151:1646-1653 (1993), Palker et al, Proc. Natl. Acad. Sci. USA 85:1932-1936 (1988), Liao et al, J. Virol. 74:254-263 (2000)). The guinea pig sera from the experiment presented in Table 4 were tested for ability to neutralize HIVRF. It was found that one sera from the C4-V3RF(A)-immunized animals (after 3 injections) had a neutralizing antibody titer of 1:40 against HIVRF, while one animal of the C4$_{E9V}$-V3RF(A)-injected animals had a neutralizing titer of 1:340 after only 2 injections. Thus, antibodies induced by the C4$_{E9V}$-V3RF(A) peptide can bind to native gpl20 and neutralize HIVRF.

Inability of the C4-E9V-RF(A) Sera to Bind to gpl20 from HIV_{IIIB}.

The V3 loop sequence of HIV_{IIIB} is different from that of HIVRF, and thus HIVRF anti-V3 neutralizing antibodies do not neutralize HIV_{IIIB}. To determine if any antibodies were generated by any of the C4-V3RF(A) variant peptides, all the mouse sera in Table 2 were tested, as were the guinea pig sera in Table 4, for the ability to bind to native recombinant HIV_{IIIB} gp120 in ELISA. Since anti-HIVRF V3 antibodies do not bind to the HIV_{IIIB} V3 loop, any binding activity of these anti-C4-V3 sera would be to the C4 region of HIV_{IIIB}, which is conserved between HIV_{IIIB} and HIVRF. No binding of any mouse or guinea pig anti-C4-V3 sera to HIV_{IIIB} gp120 was seen, indicating the inability of these peptides to induce antibodies against the native gpl20 C4 region.

Conformational Propensities of C4- V3 RF Sequence Variants in Aqueous Solution.

Next, the peptides were examined by NMR to determine whether conformational changes had been induced by amino-acid sequence alteration. It was hypothesized that specific amino-acid substitutions in the C4 segment would lead to a decrease in the tendency of this region to adopt transient helical conformations. To test this hypothesis, each of the four peptides, C4-V3RF and variants E9G, E9V and K12E, was subjected to ^{1}H NMR spectroscopy to assign resonances and to analyze nuclear Overhauser effects between hydrogen nuclei on separate residues.

Resonance assignments for nearly all ^{1}H were determined from TOCSY, DQF-COSY, and NOESY spectra by standard methods (Wuthrich, NMR of Proteins and Nucleic Acids, John Wiley and Sons, New York (1986)), and are shown in Figure 2. The value of the chemical shift for a main-chain ^{1}H, for example, the a carbon $C^{a}H$, is correlated with secondary structure in the case of proteins or well structured peptides (Wishart et al, J. Mol. Biol. 222:311-333 (1991)). Hence, strong tendencies among C4-V3RF

peptides to adopt secondary structure in solution may be manifested in chemical shift values. This was examined by calculating for each peptide the difference in chemical shift between the C-H of each residue and a shift value representing the average for all secondary structures in proteins (Wishart et al, J. Mol. Biol. 222:311-333 (1991)). In no peptide were there stretches of sequence with high or low values of the chemical shift difference that would be evidence of stable secondary structure, for example helix or β strand.

NMR parameters such as chemical shift and coupling constants are often insensitive indicators of weak preferences for particular conformations since their values are the average of the entire population, thus obscuring the contribution of a slight bias for populating certain conformations. The nuclear Overhauser effect (NOE) is often more sensitive at revealing conformational propensities because it may give rise to a unique signal, although weak, on a background consisting only of random noise. Hence, NOESY spectra of C4-V3RF and its variants were characterized to identify each signal and evaluate its relative intensity. Sequential and medium range NOEs involving main-chain NH or CaH are listed in Figure 2. These NOEs and the possible conformational propensities they represent are discussed as follows for $C4_{E9G}$-V3RF(A) and $C4_{E9V}$-V3RF(A). Variant $C4_{K12E}$-V3RF(A)K12E is discussed separately below because it was studied under different conditions.

In terms of overall conformation, all four peptides showed NOE patterns suggesting no tendency to adopt stable structure. For example, sequential daN(*i*, *i+1*) and dNN(i, *i+1*) NOEs were usually both present for each sequential pair of residues, with the former typically more intense, indicating that f and j main-chain dihedral bond angles varied and maintained on average an extended conformation (Dyson et al, Ann. Rev. Biophys. Chem. 20:519-538 (1991)). Also the absence of long range NOEs [*(i, i+5)* or greater] and the few and generally weak medium-range NOEs suggested no significant population of higher order structure.

However, the fact that some medium range NOEs were detected is evidence of propensity to adopt non-random conformations in certain regions (Dyson et al, Ann. Rev. Biophys. Chem. 20:519-538 (1991)). Although only one mixing time was used for NOESY spectra (300 ins), previous studies of a related C4-V3 RF peptide (de Lorimier et al, Biochemistry 33:2055-2062 (1994)) showed that medium range NOEs were still observable at shorter (75 and 150 ins) mixing times. Hence, the NOEs indicating medium range interactions are not likely due to spin-diffusion.

Within the C4 segment C4-V3RF and $C4_{E9V}$-V3RF(A) showed numerous medium range NOEs which are consistent with a tendency of this region to populate nascent helical conformations. The presence of contiguous or overlapping *daN(i,i+2)* NOEs from $Trp^{7}$to Tyr^{15} (C4-V3RF) and from Ile^{4} to Lys^{12} (E9V) indicates a propensity for nascent helical turns in these regions (Dyson et al, Ann. Rev. Biophys. Chem. 20:519-538 (1991), Dyson et al, J. Mol. Biol. 201:201-217 (1988)). A *dNN(i,i+2)* NOE in this region in C4-V3 RF (between Lys^{12} and Met^{14}) is also consistent with main-chain f and j dihedral angles representative of helical turns (Dyson et al, Ann. Rev. Biophys. Chem. 20:519-538 (1991)). C4-V3 RF shows three consecutive *daN(i,i+3)* NOEs from residues Val^{10} to Tyr^{15}, which is highly indicative of full helical turns. The presence of equivalent NOEs in E9V could not be ascertained due to overlap with other NOEs. However both C4-V3RF and E9V show two *dab(i,i+3)* NOEs, between Val^{10} and Ala^{13} and between Ala^{13} and Met^{14}. This type of NOE is also highly suggestive of full helical turns in these regions of C4.

Variant $C4_{E9G}$-V3RF(A)on the other hand showed no evidence, in terms of medium range NOEs, for preferential population of certain conformations in C4. This absence of medium range NOEs was not due merely to ambiguities caused by signal overlap, because there were at least five positions where an NOE was unambiguously absent in $C4_{E9G}$-V3RF(A),

but present in the parent peptide C4-V3 RF. Thus, the E to G substitution in the C4 peptide appeared to prevent helical conformer formation in the peptide.

In the V3 segment of the three peptides, C4-V3 RF, $C4_{E9G}$-V3RF(A) and $C4_{E9V}$-V3RF(A), were medium range NOEs suggesting preferred solution conformations in certain RE regions. All three peptides showed evidence of a reverse turn in the sequence Arg^{18}-Pro^{19}-Asn^{20}-Asn^{21}, where these residues comprised positions 1 to 4, respectively, of the turn. The NOE pattern consistent with a reverse turn included a weak *dNd(i,i+1)* between Arg^{18} and Pro^{19}, undetectable *ddN(i,i+1)* between Pro^{19} and Asn^{21}, weak *dad(i,i+1)* between Arg^{18} and Pro^{19}, strong *daN(i,I+1)* between Pro^{19} and Asn^{20}, and detectable *daN(i,i+2)* between Pro^{19} and Asn^{21} (Dyson et al, J. Mol. Biol. 201:161-200 (1988)). The detection of the weak *dNd(i,i+1)* NOE (Arg^{18} to Pro^{19}) suggested that a Type I turn may be the preferred conformation (Dyson et al, J. Mol. Biol. 201:161-200 (1988)).

All three peptides also showed evidence of preferred conformers at the sequence Ser^{26}-Ile^{27}-Thr^{28}-Lys^{29}. There were two consecutive *daN(i,i+2)* NOEs, between Ser^{26} and Thr^{28} and between $11e^{27}$ and Lys^{29}, as well as medium range NOEs not shown in Figure 2. The latter included a *dbN(i,i+2)* NOE between Ser^{26} and Thr^{28}, and a *dba(i,i+2)* NOE between these same residues. The conformational preferences giving rise to these NOEs did not fit a typical secondary structure, and suggested an unusual turn that placed the side-chain of Ser^{26} in close proximity to the main-chain groups of Thr^{28}. This type of conformation has been described as a kink in the context of a helical region (Osterhout et al, Biochemistry 28:7059-7064 (1989)).

A third conformational feature in the V3 segments of C4-V3RF, $C4_{E9V}$-V3RF(A) and $C4_{E9G}$-V3RF(A)occurred in the sequence $G1y^{30}$-Pro^{31}-$G1y^{32}$-Arg^{33}. In E9G the NOEs between these residues resembled the pattern described above that was consistent with a reverse turn (Dyson et al, J. Mol. Biol. 201:161-200 (1988)). This included a weak *dNd(i,i+1)* NOE between Gly^{30} and Pro^{31}, a weak *ddN(i, i-I-i)* NOE between Pro^{31} and Gly^{32}, a weak *dad(i,i+1)* NOE between $G1y^{30}$

and Pro^{31}, a strong *daN(i,i+1)* NOE between Pro^{31} and Gly^{32}, and a detectable *daN(i,i+2)* NOE between Pro^{31} and Arg^{33}. In the C4-V3RF peptide, the pattern of *(i,i+1)* NOE intensities was the same but no *daN(i,i+2)* NOE was detected between Pro^{31} and Arg^{33}. Instead a *daN(i,i+2)* NOE was detected between Gly^{30} and Gly^{32}. And in C4-E9V V3RF, both *daN(i,i+2)* NOEs, Gly^{30} to Gly^{32} and Pro^{31} to Arg^{33}, were detected. These data raised the possibility that two independent turn-like conformational preferences occurred in this region of V3. The fact that a Pro^{31}-Arg^{33} *daN(i,i+2)* NOE was unambiguously absent in C4-V3RF, and that a daN(i, *I+2)* NOE between Gly^{30} and Gly^{32} was also unambiguously absent in $C4_{E9G}$-V3RF(A), in spite of sequence identity in all three peptides, may be related to the weak intensity of these NOEs. Being close to the level of noise intensity, there is a possibility that one or both NOE signals on either side of the spectrum will not be detected, thus disallowing the given NOE to be scored as such.

Another region in V3 where conformational preferences could be inferred from NOEs occurs in residues Val^{34}-Ile^{35}-Tyr^{36}. In all three peptides NOEs were observed between the upfield methyl resonance (~0.67 ppm) of Val^{34} and the ring hydrogens, both dH and eH, of Tyr^{36}. Weaker NOEs are also seen between the downfield methyl resonance (~0.89 ppm) of Val^{34} and the ring hydrogens of Tyr^{36}. Further evidence of close proximity between the side-chains of Val^{34} and Tyr^{36} was the fact that the two methyl resonances of the former had disparate chemical shifts, compared to Val^{10}, consistent with a ring-current shift induced by the aromatic side-chain of Tyr. One peptide, C4-V3RF(A) had another NOE in this region, *daN(i,i+2)* between Ile^{35} and Ala^{37}, that was unambiguously absent in the $C4_{E9G}$-V3RF(A) and $C4_{E9V}$-V3RF(A) peptides. This observation likely represented a poorly populated conformation, perhaps related to that which gives rise to the Val^{34}-Tyr^{36} side-chain interaction, or from an independent conformational propensity.

Substitution of Lys^{12} with Glu yielded a poorly immunogenic peptide ($C4_{K12E}$-V3RF(A)) that, interestingly had solution properties different from the

other three peptides studied. Under the conditions used for NMR studies of other C4-V3 peptides, the solution of the $C4_{K12E}$-V3RF(A) peptides was highly viscous, and viscosity increased with pH in the vicinity of pH 4, implicating ionization of the Glu^{12} side-chain in this phenomenon. NMR spectra of K12E at 278 K in aqueous buffer showed a much lower signal-to-noise ratio than the other three peptides. Increasing the temperature to 318 K or decreasing the pH to 3.5 yielded improved but still inadequate signal. Suitably high signal for resonance assignment and NOE analysis was obtained at 318 K, pH *3.5, 20%* v/v trifluoroethanol *(d_3)*. Even under this condition the NOEs for the $C4_{K12E}$-V3RF(A) were less intense than for other peptides.

NOE connectivities in the C4 segment of $C4_{K12E}$-V3RF(A) (Fig. 2) show evidence of nascent helical turns in the region between Ile^3 and Gly^{11} as inferred from *dNN(i, i+2)* and *daN(i,i+2)* NOEs. The stretch from Val^{10} to Thr^{17} has two *daN(i,i+3)* and two *dab(i,i+3)* NOEs suggesting the presence of a significant population with full helical turns. Within the V3 segment only two medium range NOEs are observed, both *daN(i,i+2)*. Neither corresponds to NOEs observed in the other three peptides, but both NOEs involve residues of the Ser^{26}-Ile^{27}-Thr^{28} sequence, for which there is evidence of conformational preferences in the other three peptides. A *dbN(i,i+2)* NOE between Ser^{26} and Thr^{28}, observed in $C4_{E9V}$-V3RF(A)) and $C4_{E9G}$-V3RF(A), is also observed in the K12E peptide. Also observed are NOEs between the side-chains of Val^{34} and Tyr^{36}. Hence the conformations giving rise to these two features are at least partially preserved under the solution conditions employed for K12E. Differences in the V3 segment between K12E and all of the other three peptides include the absence of detectable daN(i, *1+2)* NOE between Pro^{19} and Asn^{21} and between Ser^{26} and Thr^{28}. The failure to detect these NOEs may be due to the overall weaker signals of this sample, or to depopulation of the relevant conformations by the solution conditions.

EXAMPLE 2

The peptides in Table 7 have been studied in groups of 5 peptides as indicated in Table 9, and each group of 5 peptides has been injected into each of three guinea pigs in Freund's complete then incomplete adjuvant. After 4 immunizations, the animals were bled, and heat inactivated serum was pooled from each animal or tested separately as indicated in Table 8, for the ability to neutralize HIV. Single numbers per group indicate that the results are those of pooled sera from the group. Individual results per animal indicate that each serum was tested individually. Table 8 shows that all the sera neutralized to varying degrees the T cell line adapted HIV isolate MN and poorly neutralized the TCLA HIV isolate IIIB. Regarding the rest of the isolates in Table 8, all of which are HIV primary isolates (89.6, BAL ADA, SF162, 5768, QH0515, PVO, JRFL, BX08, 6101, SS1196), Group C sera from C4-V3 subtype B peptides neutralized 4/11 (36%) and Group F sera from subtype B peptides neutralized 5/11 primary isolates (45%). Figure 4 shows that for the HIV CCR5 utilizing primary isolate, BAL, that the individual peptides in the 5-valent mixture absorbed out the neutralizing activity against HIV BAL to varying degrees, whereas the mixture of all the peptides completely absorbed out the neutralizing activity.

Table 8

Neutralization Of HIV-1 Isolates By Sera From Guinea Pigs Immunized With C4-V3 Clade B Peptides

Animal	Immunogen	HIVMN#	HIVIIIB#	SHIV89.6#	SHIV89.6#	HIVBAL*	ADA*	SF162*	5768*	QH0515*	PV0*	JRFL*	BX08*	6101*	SS1196*
477	A	2,258	0	96		0									
478	A	1,357	0	NA	35	0	0	90	0	0	0	0	0	0	85
479	A	4,632	68	NA		0									
480	B	1358	0	NA		0									
481	B	7,774	0	NA	27	84	0	96	0	0	0	0	0	0	0
482	B	4,241	0	62		0									
483	C	969	0	112		95									
484	C	806	0	20	97	84	0	99	0	0	0	0	86	0	0
485	C	542	0	226		80									
486	D	1,488	0	NA		0									
487	D	2,184	0	NA	98	80	0	98	0	0	0	0	94	0	0
488	D	575	0	NA		0									
489	E	3,223	0	NA		88									
490	E	NA	0	NA	255	0	0	92	0	0	0	0	0	0	0
491	E	519	0	NA		81									
492	F	NA	0	NA		NA									
493	F	910	0	NA	0	91	0	84	0	0	0	0	91	94	88
494	F	1,159	35	NA		NA									

#Assay titers are reciprocal serum dilutions at which 50% of MT-2 cells were protected from virus-induced killing as measured by neutral red uptake. *% reduction in p24 synthesis relative to the amount of p24 synthesized in the presence of corresponding prebleed samples Values >80% are positive. NA = Not available.

Table 9

G. Pig Immunization Protocol Part 2

Immunization with a group of 5 peptides			
Peptide Name	Peptide Sequence	Code	GP No.
C4-V3 peptide			
C4-V3-23.38	KQIINMWQVVGKAMYA-RPIKIERKRIPLGLGKAFYTTK	A	477,478,479
C4-V3-11.85	KQIINMWQVVGKAMYA-RPNNNTRKSINIGPGRAFYTTG	A	
C4-V3-34.29	KQIINMWQVVGKAMYA-RPNNNTRKSIQIGPGRAFYTTG	A	
C4-V3-1.481	KQIINMWQVVGKAMYA-RPNNNTRKSIHIGPGRAFYTTG	A	
C4-V3-3.323	KQIINMWQVVGKAMYA-RPNNNTRKSINMGPGRAFYTTG	A	
C4-V3-51.23	KQIINMWQVVGKAMYA-RPSNNTRRSIHMGLGRAFYTTG	B	480,481,482
C4-V3-36.29	KQIINMWQVVGKAMYA-RPNNNTRKGIHIGPGRTFFATG	B	
C4-V3-57.20	KQIINMWQVVGKAMYA-RPNRHTGKSIRMGLGRAWHTTR	B	
C4-V3-35.29	KQIINMWQVVGKAMYA-RPNNNTRKRISLGPGRVYYTTG	B	
C4-V3-46.26	KQIINMWQVVGKAMYA-RPDNTIKQRIIHIGPGRPFYTT	B	
C4-V3-69.18	KQIINMWQVVGKAMYA-RPNNNTRKSIRIGPGRAVYATD	C	483,484,485
C4-V3-72.18	KQIINMWQVVGKAMYA-RPNNNTRKGINIGPGRAFYATG	C	
C4-V3-70.18	KQIINMWQVVGKAMYA-RPNNNTRKRIRIGHIGPGRAFYATG	C	
C4-V3-62.19	KQIINMWQVVGKAMYA-RPNNNTRKSIHIGPGRAFYATE	C	
C4-V3-74.17	KQIINMWQVVGKAMYA-RPNNNTRKRMTLGPGKVFYTTG	C	
C4-V3-82.15	KQIINMWQVVGKAMYA-RPNNNTRRSIPIGPGRAFYTTG	D	486,487,487
C4-V3-113.1	KQIINMWQVVGKAMYA-RPNNNTRRSIHLGMGRALYATG	D	
C4-V3-89.14	KQIINMWQVVGKAMYA-RPSINKRRHIHIGPGRAFYAT	D	
C4-V3-85.15	KQIINMWQVVGKAMYA-RPNNNTRKSIHIAPGRAFYTTG	D	
C4-V3-122.9	KQIINMWQVVGKAMYA-RPNYNETKRIRIHRGYGRSFVTVR	D	
C4-V3-170.6	KQIINMWQVVGKAMYA-RPNNNTRRSVRIGPGGAMFRTG	E	489,490,491
C4-V3-146.8	KQIINMWQVVGKAMYA-RPGNNTRRRISIGPGRAFVATK	E	
C4-V3-163.7	KQIINMWQVVGKAMYA-RLYNYRRKGIHIGPGRAIYATG	E	
C4-V3-125.9	KQIINMWQVVGKAMYA-RPNNNTRRRISMGPGRVLYTTG	E	
C4-V3-162.7	KQIINMWQVVGKAMYA-RPGNNTRGSIHLHPGRKFYYSR	E	
C4-V3-396.2	KQIINMWQVVGKAMYA-RPNNNTRRNIHIGLGRRFYAT	F	492,493,494
C4-V3-144.8	KQIINMWQVVGKAMYA-RPDNNTVRKIPIGPGSSFYTT	F	
C4-V3-365.2	KQIINMWQVVGKAMYA-RPSNNTRKGIHLGPGRAIYATE	F	
C4-V3-513.2	KQIINMWQVVGKAMYA-RPSNNTRKGIHMGPGKAIYTTD	F	
C4-V3-1448.1	KQIINMWQVVGKAMYA-RPGNTTRRGIPIGPGRAFFTTG	F	

It is important to be able to use T helper determinants with the V3 portion of the peptides shown in Table 7, both to expand the T helper activity in the immunogen, and in case any of the T helper peptides should be found to have any deleterious effects in the course of human trials. For example, it has recently been found *in vitro* that in culture of HIV and T cells, that the C4 portion of the C4-V3 peptide can augment HIV induced syncytium formation. However, peptides of this general design have been studied *in vitro* in HIV-infected humans (AIDS 12: 1291-1300, 1998) and no subjects developed a ≥ 10 fold change in plasma HIV RNA levels from baseline. Moreover, the primary use of these peptides is as an immunogen in HIV- subjects as a preventive vaccine, and not in doses that one would consider for therapy, which would be in milligram amounts daily. A T helper determinant from HIV gag, termed GTH1 with the sequence of Y K R W I I L G L N K I V R M Y S has been conjugated to the V3 of HIV MN and found to induce anti-HIV MN titers of 1:3200. Similarly, GTH1 conjugated to a V3 sequence of a HIV primary isolate DU179 induced antibodies that neutralized HIV MN (1:192) and neutralized the HIV primary isolate JR-FL (90% p24 reduction in PBMC cultures). Thus, the GTH1 T helper sequence can substitute for the C4 sequence in the peptides in Table 7.

Finally, a panel of monovalent serum from individual guinea pigs immunized with each of the peptides in Table 7 has been screened. Whereas most of the peptides in the list only induced neutralizing antibodies that neutralized 0 to 6 out of 19 primary isolates, 5 peptides were found that neutralized from 14 to 19 out of 19 primary isolates tested. These peptides were C4-V3 36.29, C4-V3 34.29, C4-V3 62.19, C4-V3 74.17, and C4-V3 162.7. The sequences of these peptides are all listed in Table 7.

Thus, sufficient breadth has been observed both in mixtures of C4-V3 peptides and in select individual peptides for the immunogen to be practical with regard to induction of neutralizing antibodies against HIV primary isolates. By performing the same immunization studies with the similarly

designed HIV subtype (clade) C peptides in Table 6, that a similar immunogen(s) can be developed for HIV subtype C viruses.

While individual peptides can be used to achieve the breadth of neutralizing activity needed to protect against HIV primary isolates, advantageously, mixtures of multiple peptides are used, such as the combination of group C, or group F or the combination of C4-V3 36.29, C4-V3 34.29, C4-V3 62.19, C4-V3 74.17, and C4-V3 162.7 peptides described above.

* * *

All documents cited above are hereby incorporated in their entirety by reference.

WHAT IS CLAIMED IS:

1. A composition comprising a multiplicity of immunogenic peptides comprising a first and a second component,

said first component comprising a T-helper epitope,

said second component comprising residues of the V3 domain of gp120 and including a B cell neutralizing antibody epitope.

2. The composition according to claim 1 wherein said first component is an a human immunodeficiency virus (HIV) T helper epitope.

3. The composition according to claim 2 wherein said first component comprises residues of the C4 domain of HIV gp120.

4. The composition according to claim 3 wherein said first component comprises at least 16 contiguous residues of the C4 domain of HIV gp120.

5. The composition according to claim 4 wherein said first component comprises residues 421 to 436 of the C4 domain of HIV gp120.

6. The composition according to claim 2 wherein said first component comprises residues of HIV p24 gag.

7. The composition according to claim 6 wherein said first component comprises GTH1 (residues 262-278 of HIV gag).

8. The composition according to claim 1 wherein said first component is a non-HIV T helper epitope.

9. The composition according to claim 1 wherein said second component comprises at least 23 contiguous residues of the V3 domain of HIV gp120.

10. The composition according to claim 9 wherein said second component comprises residues 297 to 322 of the V3 domain of HIV gp120.

11. The composition according to claim 1 wherein said first component comprises at least 16 contiguous residues of the C4 domain of HIV gp120 and said second component comprises at least 23 contiguous residues of the V3 domain of HIV gp120.

12. The composition according to claim 11 wherein said first component comprises residues 421 to 436 of the C4 domain of HIV gp120 and said second component comprises residues 297 to 322 of the V3 domain of HIV gp120.

13. The composition according to claim 1 wherein said second component is linked C terminal to said first component.

14. The composition according to claim 1 wherein said first component is linked to said second component via a linker.

15. The composition according to claim 1 wherein said composition comprises at least 5 immunogenic peptides.

16. The composition according to claim 15 wherein said - composition comprises C4-V3 36.29, C4-V3 34.29, C4-V3 62.19, C4-V3 74.17 and C4-V3 162.7 from Table 7.

17. The composition according to claim 15 wherein said composition comprises at least 10 immunogenic peptides.

18. The composition according to claim 17 wherein said composition comprises at least 25 immunogenic peptides.

19. The composition according to claim 1 wherein said composition further comprises a carrier.

20. The composition according to claim 1 wherein said composition further comprises an adjuvant.

21. A composition comprising at least one peptide from Table 6 or Table 7 and a carrier.

22. The composition according to claim 21 wherein said composition further comprises an adjuvant.

23. The composition according to claim 1 wherein second components are selected so as to be representative of higher order structural motifs present in a population, which motifs mediate V3 functions in the course of envelope mediated HIV interaction with host cells.

24. The composition according to claim 23 wherein said composition comprises about 25-30 immunogenic peptides the second components of which are selected so as to be representative of infected individuals within a subtype.

25. The composition according to claim 1 wherein at least one of said first components comprises the sequence KQIINMWQVVGKAMYA.

26. A method of inducing the production of neutralizing antibodies in a patient comprising administering to said patient an amount of the composition according to claim 1 sufficient to effect said production.

27. A nucleic acid encoding a peptide in Table 6 or Table 7.

28. A formulation comprising at least one nucleic acid sequence encoding said composition of claim 1.

29. A method of inducing the production of neutralizing antibodies in a patient comprising administering to said patient an amount of said formulation according to claim 27 sufficient to effect said production.

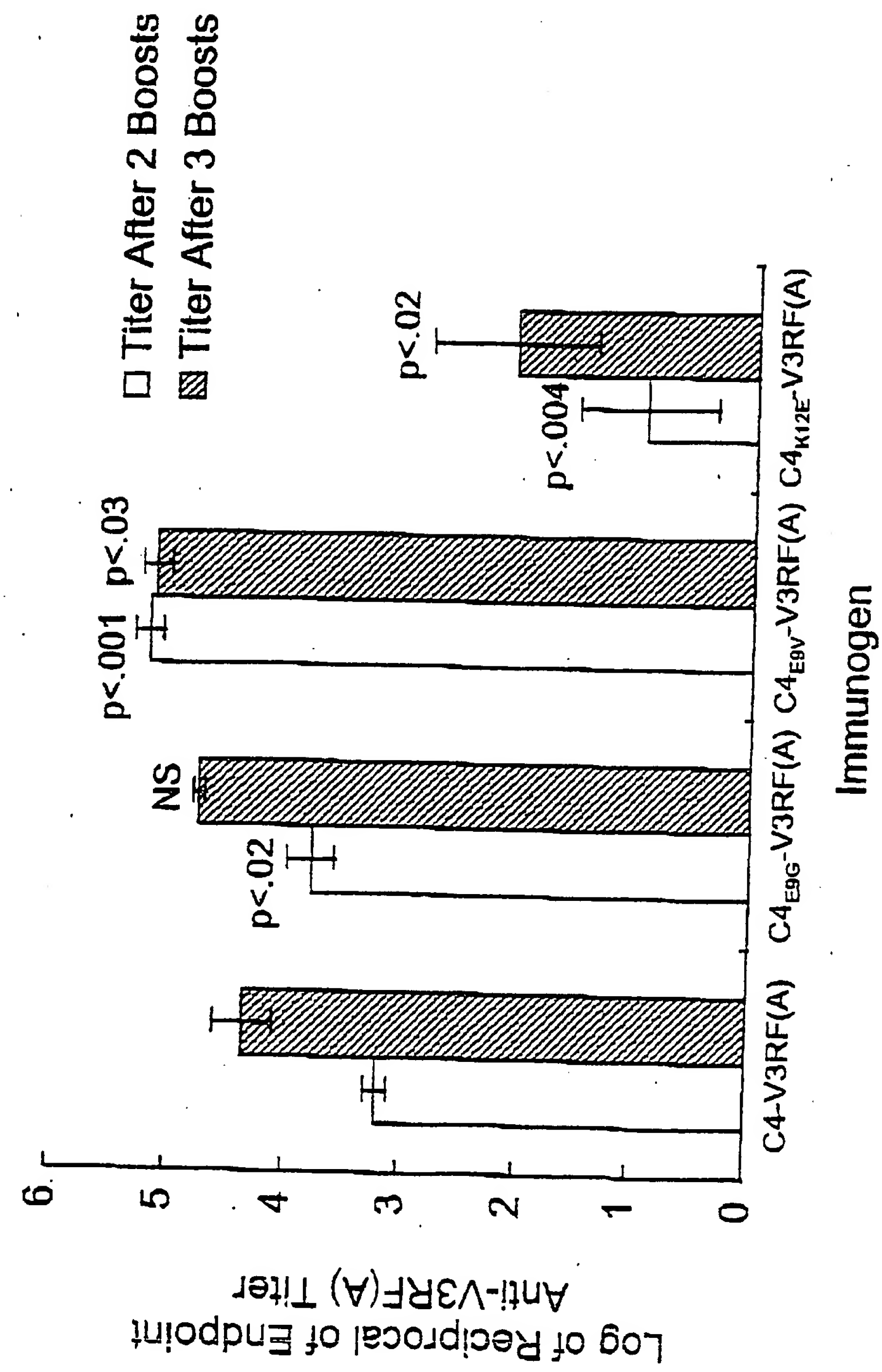
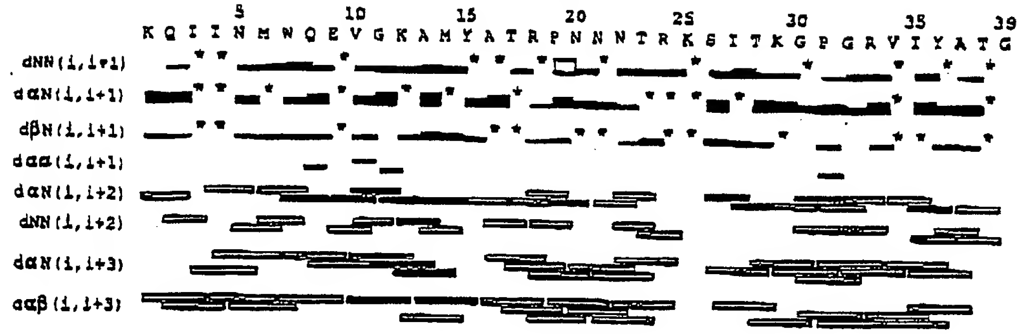
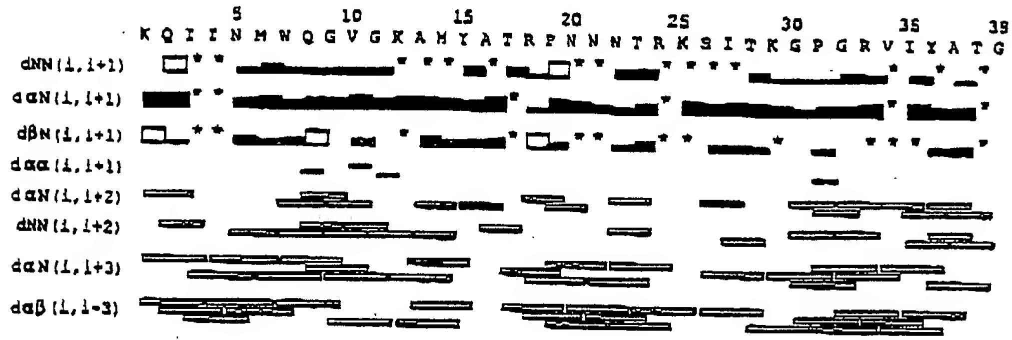

Titer After 2 Boosts
Titer After 3 Boosts
Log of Reciprocal of Endpoint Anti-V3RF(A) Titer
0
1
2
3
4
5
6
C4-V3RF(A)
$C4_{E9G}$-V3RF(A)
$C4_{E9V}$-V3RF(A)
$C4_{K12E}$-V3RF(A)
Immunogen
$p<.02$
NS
$p<.001$
$p<.03$
$p<.004$
$p<.02$

Figure 1

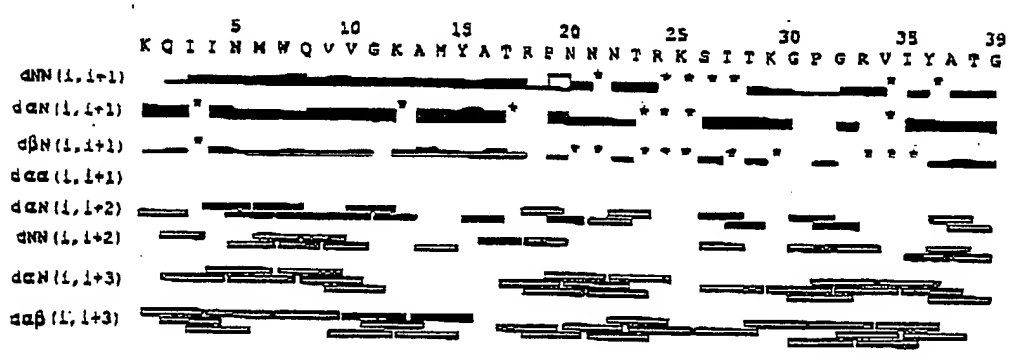
C4-V3 RF



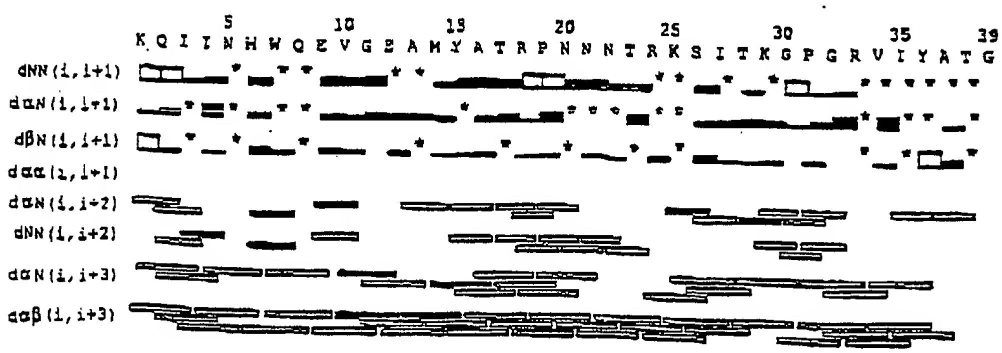
E9G



E9V



K12E



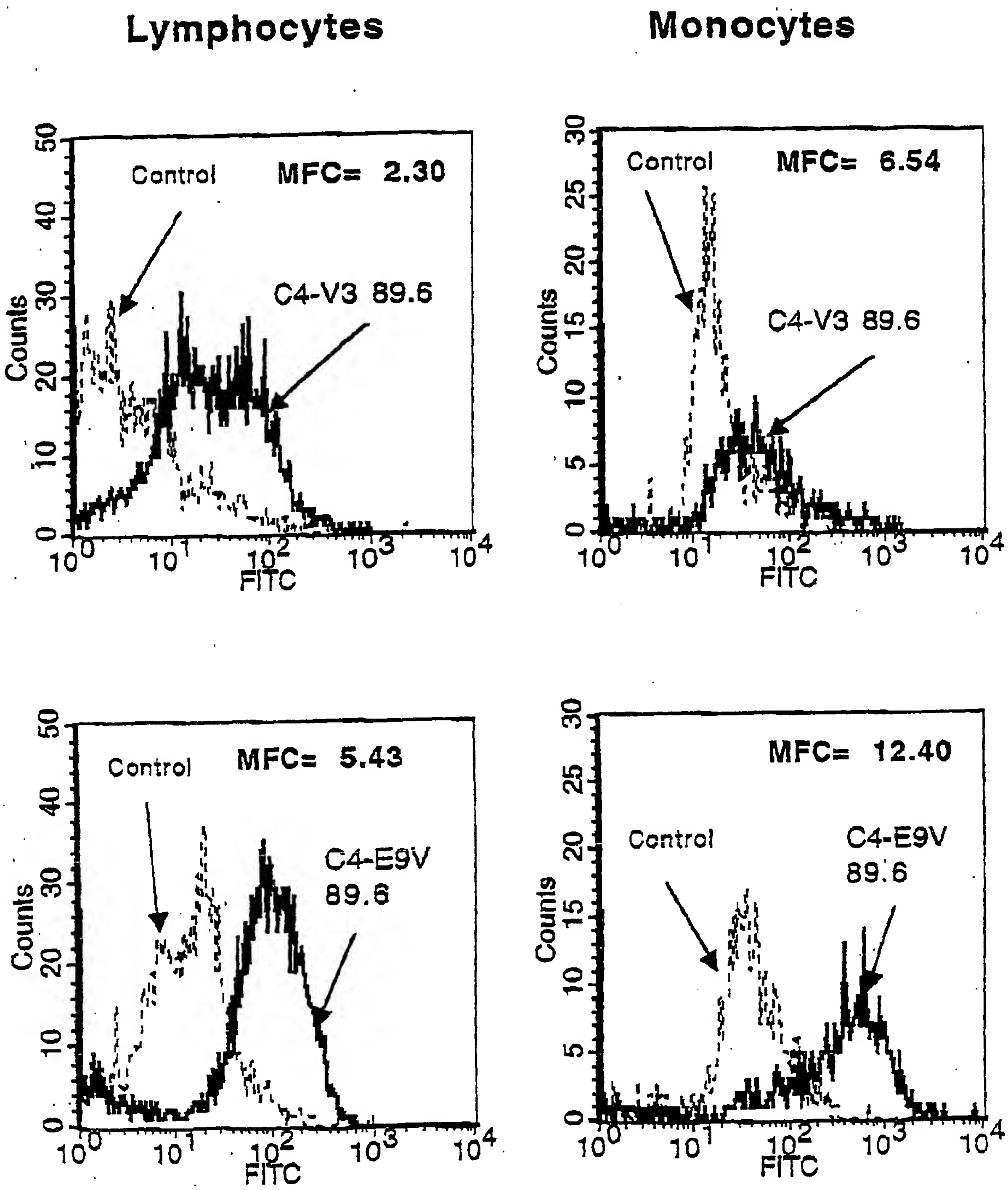
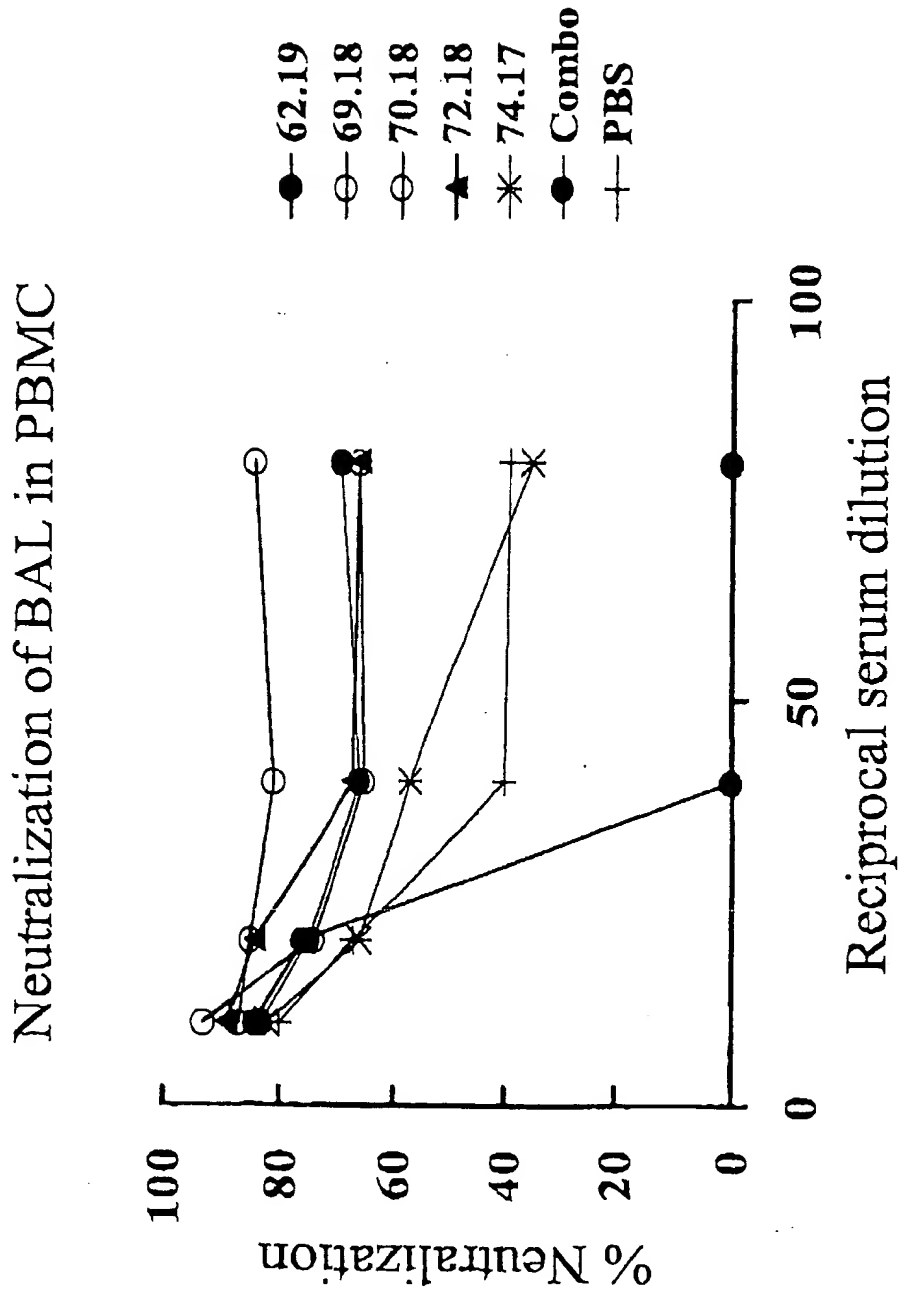

Lymphocytes
Monocytes
Control
MFC= 2.30
C4-V3 89.6
Counts
FITC
Control
MFC= 6.54
C4-V3 89.6
Counts
FITC
Control
MFC= 5.43
C4-E9V
89.6
Counts
FITC
MFC= 12.40
Control
C4-E9V
89.6
Counts
FITC

Figure 3


Neutralization of BAL in PBMC
% Neutralization
100
80
60
40
20
0
0
50
100
Reciprocal serum dilution
62.19
69.18
70.18
72.18
74.17
Combo
PBS